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The Council For Tobacco Research – U.S.A., Inc.

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Application for Research Grant
(Use extra pages as needed)

Date: 12/14/73

Case No. 246

1. Principal Investigator (give title and degrees):

Ted P. McDonald, Ph.D., Research Associate Professor

2. Institution & address:

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3. Department(s) where research will be done or collaboration provided:

University of Tennessee Memorial Research Center
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4. Short title of study:

Tobacco Smoke and Platelet Function

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Acute myocardial infarction is a major cause of death and individuals who smoke have an increased risk of cardiac and vascular disorders as compared with nonsmokers. Most of the studies reported in the literature are plagued by small samples, inappropriate controls, use of hospitalized patients and often present conflicting results. The intent of this proposal is to carry out a controlled laboratory study in rats and human volunteers on the effects of tobacco smoke and nicotine on: 1) platelet function, including adhesion, aggregation, clot retraction, and plasma factors; 2) platelet production, including peripheral platelet counts, platelet life span, platelet size, and changes in the bone marrow megakaryocyte population; 3) chemical alterations of platelet mucopolysaccharides, hexosamines and protein content; and 4) determination of thrombopoietin levels in serum from treated animals and human volunteers with the goal of adding to our present knowledge of the involvement of platelets in coronary heart disease and clarifying some of the conflicting and poorly substantiated data present in the literature.

8. Brief statement of working hypothesis:

Coronary insufficiency may be preceeded by impairment of blood flow through the vessels near or in the heart itself. This impairment may be due to actual alterations in the endothelial lining of the blood vessels (such as increased atherosclerosis among smokers)[1] or to the sticking of platelets either to one another or to vessel walls, which could partially or completely occlude blood vessels. Increased atherosclerotic vessels are jagged and lose their silicone-like properties,[2] thus increasing the prossibility of rupture and concomitant aggregation of platelets into clumps or thrombi. If platelet clumps are not broken apart they can lodge in blood vessels, stopping the flow of blood. Therefore, one important aspect of determining how smoking causes an increased risk of heart attacks is to examine the effects of smoking on the formed elements of the blood which are intimately involved in vascular blockages - the platelets. Several methods of inquiry will be used to examine the effects that smoking and nicotine have on blood platelets.

9. Details of experimental design and procedures (append extra pages as necessary)

A. Literature review, significance, and reasons for study.

More people die as a result of heart attacks every year than any other disease.[3] Smokers have a significantly higher incidence of myocardial infarctions[1,5] related vascular disorders such as Burger's disorders,[4] coronary artery disease,[1,6,7] carcinoma of the lung[8] and other types of malignancy,[9] as well as chronic bronchitis and pulmonary emphysema[6] as compared to nonsmokers. One study indicated that nonsmokers who are in close association with smokers often inhale almost as much cigarette smoke as a smoker.[10] The children of parents who smoke suffer from the toxic effects of cigarette smoke all of their lives whether or not they become smokers themselves.[10] Mothers who smoke during pregnancy block oxygen transport - in direct relation to the smoking habit - across the placental membrane to the fetus which results in an increased number of still-born babies or children with retarded growth and/or development.[11,12]

(1) Tobacco smoke and platelet activity.

Cigarette smoke has been shown to cause an accelerated thrombus formation *in vitro*,[13] supporting the finding that smokers have an increased thrombotic tendency. Sixty human subjects both male and female were used with ages ranging from 17 to 68 years. All subjects were patients in a private hospital and all were habitual smokers. Each patient served as his own control and used his own cigarette brand. Blood samples were taken before smoking and 20 min after smoking 2 cigarettes; 34 out of 60 habitual smokers showed accelerated thrombus formation.

Engelberg and Futterman[14] later repeated this work using a similar experimental design, but with a larger sample size (147 individuals). Again, all subjects were habitual smokers and were either hospital patients or staff at the hospital. The reasons for hospitalization of numerous test subjects were not indicated and each person again served as his own control. The results of this study showed 132 of the 147 individuals had an accelerated thrombus formation after smoking 2 cigarettes.

Mustard and Murphy[15] reported that smoking decreased platelet survival and increased platelet turnover, thereby shortening the platelet life span. In this work only 7 human subjects were used and each had a history of some type of vascular or heart malady: myocardial infarction, cerebrovascular accidents or disorders and all were heavy smokers, i.e. greater than 1 pack per day. Since O'Brien *et al.*[16] found that nonsmoking patients suffering from myocardial infarctions had more active platelets (larger size and greater adhesion) than normal nonsmoking subjects, the above studies are in question.

Platelet adhesiveness has been reported to be enhanced after smoking in studies by Ambrus and Mink,[17] Ashby *et al.*[18] and by El-Ebrashy *et al.*[19]; whereas, Mustard and Murphy[5] and Murchison and Fyfe[20] found no such increase after smoking.

A decreased platelet count and decreased blood coagulation time in patients after smoking a single cigarette was reported by Shimamato.[2] This decrease was postulated to be due to damage of the endothelial silicone-like property of the blood vessel. Recently, Hawkins[21] also demonstrated that the blood of smokers had a decreased coagulation time as compared to nonsmokers, as well as increased rates of initial clot formation and clot retraction. Platelets from individuals who smoked were more active than those of non-smokers when aggregated with ADP. The clot formation and clot retraction data are based on thromboelastography which can measure tensil strength of clots and the rate of clot formation. A different technique has been used recently to demonstrate increased clot retraction of rat blood platelets after nicotine treatment.[22,29] This technique allowed a determination of weight changes of the retracted clot and seems to be a better method for determining increased function of platelets.

Levine[23] recently presented evidence that smoking a single cigarette caused an increase in the platelet's response to a standard aggregating stimulus in human volunteers. The response appears to be related specifically to the inhaling of tobacco smoke, since it was not observed following the smoking of lettuce leaf filled cigarettes.

(2) Nicotine and platelet activity.

Nicotine is frequently considered to be the causative agent of tobacco smoke in the development of coronary and vascular disease, although this association has not been conclusively proven. Many investigators have examined the effects of nicotine on numerous tissues of the body, but there are relatively few studies on the effects of nicotine on blood platelets.

Wenzel and Singh[24] have shown that intravenous injections of nicotine depressed coagulation time in rabbits. Epinephrine likewise decreased coagulation time and an injection of nicotine and epinephrine given together acted synergistically to decrease coagulation time. Earlier work by Wenzel *et al.*[25] demonstrated that both oral and intravenous administration of nicotine to rabbits resulted in decreased coagulation time. These findings seem to indicate that the route of administration of nicotine into the body does not affect the action of nicotine on coagulation time.

Recently, McDonald and Clift[26] described increased platelet aggregation in rats after oral nicotine administration when compared to aggregation of platelets from control rats. The increased aggregation appeared to be due to both an alteration of some plasma factor and to changes in the platelets themselves. More experiments are needed using larger samples and standardized platelet numbers to determine the mechanism of alteration.

Werle and Schievelbein[27] suggested that platelet aggregation was proportional to the concentration of nicotine used. Unfortunately, in order to show platelet alterations, high concentrations of nicotine had to be administered. They also found that nicotine and ADP enhanced platelet aggregation when added simultaneously. The aggregometer used in these studies required the use of a manual stirrer which could rupture platelets or produce uneven distributions resulting in false aggregation data. By use of a Chrono-log aggregometer with continuous automatic mixing (described in the following methods section), one could more accurately measure and determine the aggregation of platelets exposed to various substances.

One of the most widely used tests of platelet alteration after smoking is platelet adhesion.[28] The techniques which are employed have a high degree of variability so the results are questionable. No conclusive data are present in the literature concerning the effect of nicotine on platelet adhesion. Therefore, further investigations, using refined techniques, need to be performed in order to clarify the effects of nicotine on platelet adhesion.

Another method of measuring platelet function is by use of clot retraction. An increase in clot retraction as determined by the weight of the retracted clot has been shown to be a measure of platelet function. McDonald *et al.*[29] have recently demonstrated that rats exposed to nicotine (both oral and intravenous) have increased clot retraction (which results in a smaller clot) and were therefore more functional as compared to nontreated controls. One can conclude, therefore, that nicotine ingestion definitely alters certain platelet functions.

(3) Smoking vs nicotine effects

Isaac and Rand[30] reported that the amount of nicotine extracted from a single cigarette was concentrated in the plasma of a smoker by a factor of 1.5 to 2.5 depending on the nicotine content of the cigarette. Although the amount of nicotine in the plasma was reduced by one-half 30 min following cessation of smoking, a smoker could accumulate higher and higher concentrations of nicotine by continued smoking.

Smoking and nicotine injections have both been shown to decrease clotting time in dogs. However, nicotine alone did not alter platelet counts or fibrinogen levels but smoking did.[31] Since tobacco components are altered while the cigarette burns,[32] it is very probably that oral or intravenous administrations of nicotine would not simulate tobacco components found in the burning cigarette, thus the necessity of studying the effect of smoke rather than nicotine.

These findings strongly suggest that while nicotine may account for certain physiological changes associated with smoking, cigarette components other than nicotine may be responsible for various alterations in platelet activity that could lead to heart and vascular malfunctions. Therefore, a comparative study of the effects of nicotine and tobacco smoke on blood platelets needs to be performed.

(4) Need for laboratory study.

Most of the studies to date concerning platelets and smoking have been limited to platelet functions (adhesion, aggregation and clot retraction) and have not dealt with other parameters of platelet activity such as their production and chemistry. In general, results of smoking studies in the literature have considerable experimental error. The majority of studies concerning the effects of smoking on blood platelets have been performed on human subjects, who for the most part, have been categorized depending on their smoking habits as a nonsmoker, a light smoker (1 pack or less/day) or a heavy smoker (more than 1 pack per day). Test subjects are required to smoke cigarettes either *ad libitium* or a predetermined number for a designated period of time. After smoking, blood is taken and various platelet activities are analyzed. Most of the studies use only smokers and therefore have no suitable control group. In addition, to compound the error, subjects are often hospital patients suffering from one or more types of heart and/or vascular disorders in which altered platelet functions have been found.

Futhermore, the use of human subjects also prohibits the investigator from controlling the duration of the cigarette puff, thus varying the doses among the smokers. A standard puff of cigarette smoke, as defined by the British and American tobacco council, consists of a two second puffs of 35 ml of smoke pulled through a cigarette once each minute down to a 30 mm butt length.[30,33] It seems possible that some smokers may obtain roughly 30-50 times this amount of smoke and other smokers may obtain less thus varying the amounts of smoke constituents such as the amount of nicotine per cigarette.[30] If alterations in platelet function, production and chemistry are found as compared to controls, then for the first time one can accurately attribute these changes to treatment rather than to biological variations in health, diets, emotions, different doses, as well as the noxious effects of the various types of pollution than human subjects encounter.

B. Rationale and Methods

It is planned to study rats after exposure to tobacco smoke or nicotine treatment for altered platelet function (platelet adhesion, aggregation, clot retraction, and plasma factors); for changes in platelet production (peripheral platelet counts, platelet life span, size and megakaryocyte numbers); for chemical alterations of platelets (mucopolysaccharide content, hexosamine values, and amounts of protein); and for thrombopoietin content of sera. In addition, a study of platelets in human volunteers is planned.

(1) Methods of Exposing Animals

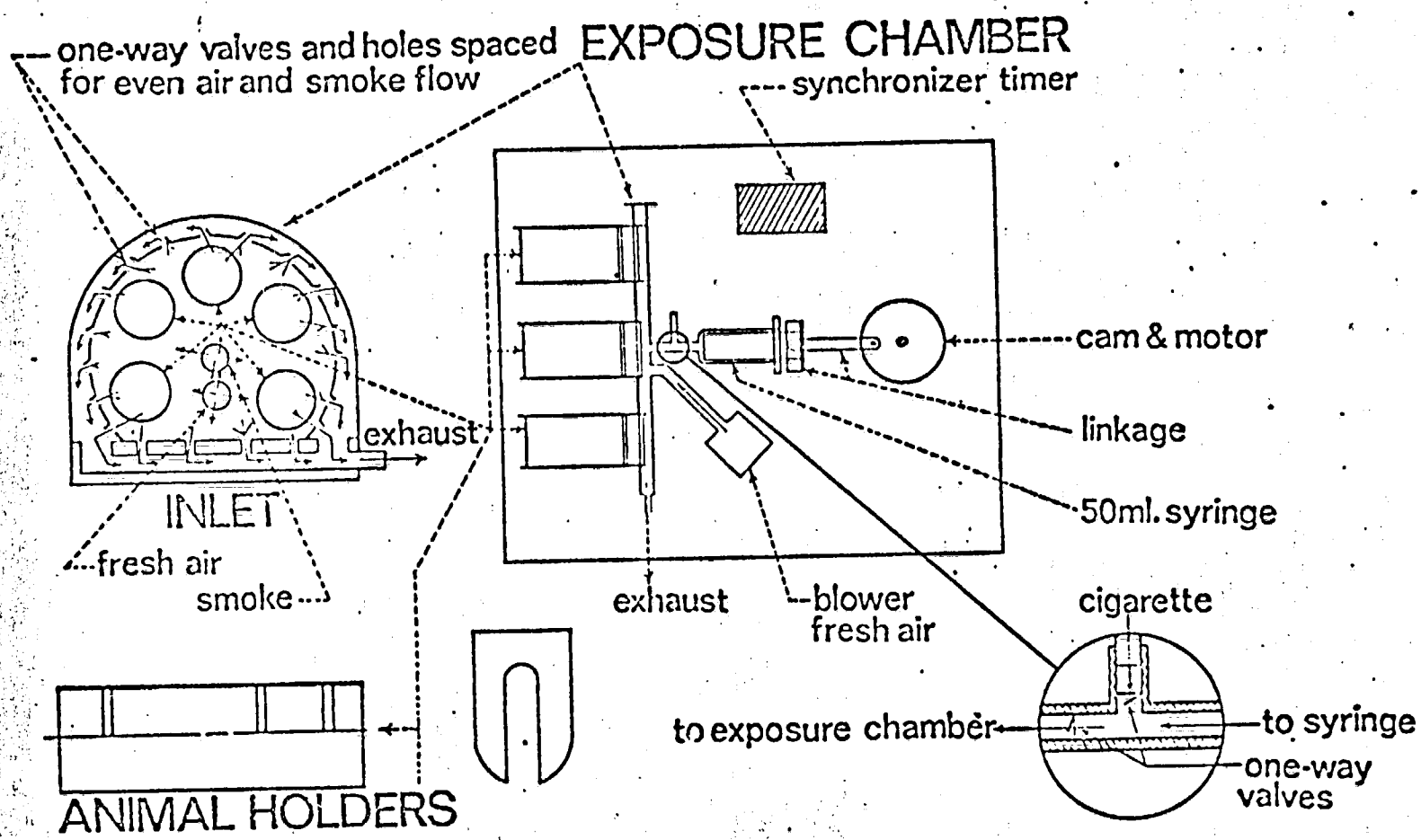
(a) Smoking Machine

Smoke will be administered to male Sprague-Dawley rats (200-350 gm) by use of a small "Walton" smoking machine purchased from Process Instrument, Inc., 1943 Broadway, Brooklyn, NY. A machine (see attached figure) which is a modification of several other machines will be used until the new machine is available.[34,35] The Engineering Mechanics Department at the University of Tennessee designed and built this machine according to our specifications.[36] The machine delivers a standard puff[30,33] of cigarette smoke each minute to rats with a minimum amount of smoke dilution. This machine will simultaneously expose 5 rats to standard puffs of cigarette smoke with roughly a 1:7 dilution of smoke with air. Rats are exposed to fresh smoke for 20 seconds after which a blower exhausts the breathing chamber and continues to give the rats fresh air for 30 seconds. The blower then shuts off and coasts to a stop before the next puff cycle begins.

Two types of control groups will be used to insure that the stress of the machine and/or restraint devices do not induce any platelet alterations. One group will remain in their normal cages undisturbed for the 10 day exposure period. Another group of controls will be sham-smoked for as long as the longest treatment groups.

At present, our treatment groups include exposure to either 2, 8, or 16 cigarettes/day for 10 days. These represent light doses in comparison to those obtained by a heavy smoker (> 1 pack/day). These doses may need to be modified in accordance with the increased efficiency of the "Walton" machine.

Since the normal platelet life span in rats is about 4.5 days,[39] the rats will be exposed for 10 days to insure that all the platelets examined have been produced under smoking conditions. A standard reference cigarette (1R1 purchased from the University of Kentucky - Tobacco and Health Research Institute) will be used for all experiments to insure a consistent tobacco composition, thus greatly reducing the variability between groups of cigarettes.



(b) Nicotine Administration

Nicotine (nicotine alkaloid, Sigma Chemical Co.) will be injected intravenously (10-160 µg/kg body wt) or given to rats in their drinking water for 2 weeks prior to assay at concentrations of 25 mg/L, 12.5 mg/L and 0 mg/L. Every day fresh nicotine-water will be prepared and 400 ml placed on rat cages (2 rats/cage). The amount of liquid remaining in the bottles each day will be used to calculate the dose on a mg nicotine/kg body wt/day basis.

(2) Platelet Function Studies

(a) Platelet Adhesion

The adherence of platelets to surfaces such as the endothelial lining of blood vessels is referred to as adhesion. Although platelets normally adhere to subendothelial layers of vessel walls at sites of injury to prevent blood loss, adherence to vessel walls, other than for reasons of maintaining hemostasis, can block the flow of blood. Increased platelet adhesion has been directly correlated with acute venous and arterial thrombosis.[40,41] Since individuals who smoke have an increased occurance of various types of vascular occlusions, one effect of smoking might be altered platelet adhesiveness.

In this work a modification of Salzman's method[42] will be used to determine platelet adhesion. Rats will be anesthetized with ether and 7-8 ml of blood drawn from the dorsal aorta into a 12 ml plastic syringe containing 10 U of heparin per ml of blood. The excess blood over 5 ml will be expressed into a separate tube for routine analysis (platelet count, white blood cell (WBC) count, hematocrit and red blood cell (RBC) count) and utilized for the initial platelet count. The remaining 5 ml will be expressed through a 17.2 cm polyvinyl tubing filled with glass beads (3M Superbrite beads 100-5005) at the rate of 1 ml/15 seconds into a 15 ml EDTA tube for the final platelet count. The percentage of platelets remaining in the glass-bead-filled tube, referred to as the percent platelet adhesiveness, will be calculated by substracting the final platelet count from the initial count divided by the initial platelet count times 100:

$$\text{Percent Platelet Adhesiveness} = \frac{\text{Initial count} - \text{Final count}}{\text{Initial count}} \times 100$$

By use of this procedure the platelet adhesiveness index for rats is usually between 65-85%. In order to adequately measure an increase in adhesiveness due to smoking one needs to reduce the normal values to around 50%. The adhesiveness index can be reduced by shortening the polyvinyl tube length and/or increasing the flow rate of the blood over the glass beads. If smoking causes significant increases in adhesiveness over nontreated controls, then one should be able to measure these changes using such a modified technique.

(b) Platelet Aggregation

The adherence of platelets to one another (aggregation) is another important mechanism in maintaining normal hemostatic conditions within the body. Under certain conditions platelets can aggregate and form a thrombus or a clot in blood vessels, which can occlude the vessel and many times lead to a fatal condition. Several substances are known to cause platelets to aggregate: adenosine diphosphate (ADP), epinephrine, collagen, and others. Platelets normally contain ADP, so when a platelet ruptures it releases ADP causing other platelets to rupture which cascades into a platelet aggregate.[41] Nicotine is known to cause increased levels of epinephrine in the plasma which might account for the increased platelet aggregation among smokers.[5] Levine[23,43] recently reported that aggregation induced by ADP, epinephrine, or collagen was more pronounced in platelets of cigarette smokers than in nonsmokers.

A Chrono-log Platelet Aggregometer attached to a Chrono-log model 702 recorder will be used to determine platelet aggregation;[44] ADP, collagen, and epinephrine will serve as the aggregating agents. Platelet rich plasma (PRP) and platelet poor plasma (PPP) will be prepared in the following manner: 8 ml of blood will be drawn from the abdominal aorta of each rat into 12 ml plastic syringes containing 80 U of heparin. The blood will undergo differential centrifugation (107 x g) for 30 min. at 22°C to obtain the PRP and to separate the platelets from the WBC and RBC. The PPP will be prepared by a more rapid centrifugation (760 x g) of the remaining blood for 20 min. Platelet suspension counts using an Electrozone Celloscope will be made so that the concentration of aggregating agent/platelet can be kept constant throughout experiments. A 0.5 ml sample of the PRP and the PPP will be placed into 0.312 inch diameter siliconized cuvettes along with a small teflon stir-bar. While in the aggregometer the platelet preparations will be stirred at a constant rate (1200 rpm) and maintained at 37°C.

Since the operating principle of the aggregometer is based on the interception of light on a photocell (as in a spectrophotometer), the PRP will cause a decrease in transmittance; whereas, the PPP will allow an increase. After determining 10% and 90% transmittance with PRP and PPP, an aggregating agent will be pipetted into the cuvette in order to start the aggregation. A tracing of approximately 5 min will be recorded and the percent aggregation at one, two and three min after the introduction of the agent will be measured by the change in light transmission. The rate of aggregation, the relative size of the aggregates, and the percent aggregation can be determined from the curve which will be plotted. This will enable one to accurately determine some of the effects that smoking might have on platelet aggregation.

(c) Clot Retraction

ADP release and subsequent release of platelet factor-3 from platelets cause a lengthy series of coagulation reactions which ultimately end in the formation of a clot sealing off the ruptured vessel. The clot then retracts through the action of the contractile-like protein, thrombosthenin, forming the final hemostatic plug. Normally, this clot will be dissolved and removed from the circulation.[22] The clots which form in vessels that are not in response to normal hemostatic maintenance and are not dissolved can result in vascular blockage. It is the formation of this latter type of clot which is primarily responsible for myocardial infarctions among smokers. In a recent study, nicotine has been shown to increase clot retraction in rats.[29] However, no studies apparently have been performed concerning the effect of tobacco smoke on clot retraction.

Clot retraction studies will be done according to the method outlined by McDonald *et al.*[29] PRP will be diluted with PPP to give platelet concentrations of 400,000; 200,000; 100,000; 50,000; 25,000; 12,500; and 6,250 platelets/mm^3. To 0.2 ml of each of the above PRP (in plastic Kahn tubes) 0.2 ml of PPP containing 0.1 volume of 0.33 M $CaCl_2$ will be added. In order to initiate clot formation, 0.05 ml of thrombin (50 U/ml) will be added to each tube and the tubes incubated for 2 hrs in a 37°C water bath. After 10 min the clots will be loosened from the walls of the tube by agitation and returned to the bath. After incubation the clots will be weighed to determine the extent of retraction. If smaller than normal clots are found, then the clot has retracted to a greater extent and is, therefore, more functional. Reduced weight of clots (as compared to normal controls) indicates that the platelets are more active than normal and hence more likely to be involved in vascular occlusions.

(d) Plasma Factors

Previous work by the applicant[29] has shown that the effects of nicotine on clot retraction of rat blood platelets were not directly on platelets. It was concluded that the enhanced clot retraction observed in the blood of nicotine-treated animals was due to a plasma factor and not to changes in the platelets themselves.[29]

As a corollary to the work on platelets, a secondary effort will be focused on the isolation of the plasma factor. Fibrinogen may be a necessary co-factor for ADP-induced platelet aggregation and can be isolated by the use of newer techniques from plasma of animals and humans after smoking.[59] The extracted fibrinogen can then be used in the determination of specific effects on platelets. In addition, other plasma proteins will be partially purified by electrophoresis and other methods for testing in vitro.

(1) Detection and quantification of fibrinogen in blood plasma

The method for the detection and quantification of fibrinogen complexes, fibrinogen itself and fibrinogen derivatives in plasma is as follows: columns used are 1 x 30 cm and gel employed is Bio-Gel 5M (Bio-Rad) with an average gel height of 29 cm. Tris saline buffers, [14.5 grams tris (hydroxymethyl)aminoethane, 35 g sodium chloride, pH adjusted to 7.6 with 1 N HCl and made up to 2 liters] will be used both for column equilibration and for gel filtration. All procedures are run at room temperature to avoid difficulties with cryoprotein precipitation. Columns are packed in the conventional manner and then calibrated with known normal plasma with the column flow rate at 12 ml an hour. "Normal" plasma is eluted, as described below, and column gel content adjusted so that under standard flow or elution conditions, fibrinogen patterns are symmetrical at 10 ml elution volume. If treated carefully, and washed scrupulously, a single calibrated column may be used for 20-50 determinations before requiring repacking and recalibration. The fact that a number of normal plasmas are necessarily included in the assay samples allows a continual check on the state of column calibration.

Plasma samples (1 ml) are applied to the column over a layer of 40% sucrose (0.5 ml) to sharpen the original boundary zone. Buffer is carefully layered over the column when the plasma layer has sunk just below the top gel layer and the buffer feed is then connected. Effluent samples (0.8 ml) are collected and assayed for fibrinogen by (a) thrombin-clottable protein[60] and/or (b) by radial immunodiffusion[61] using specifically-absorbed fibrinogen antiserum. Effluent fibrinogen concentration is plotted against effluent volume and the plot compared with that of a normal plasma of identical fibrinogen concentration (a computer program which is now under development will be used to quantify this last step.)[59]

This method of fibrinogen determination in its present form is exceptionally laborious to perform and is technically difficult. However, the applicant has several years experience using column chromatography methods and the principle or co-investigator plans to spend sufficient time in Dr. A. P. Fletcher's laboratory in St. Louis to develop the skills necessary to obtain satisfactory and reproducible results before attempting these studies in this research center.

(2) Isolation of plasma proteins

Preliminary studies will be conducted to determine whether or not the factor that increases platelet function and found in plasma of rats after smoking or nicotine treatment is also found in serum and urine of human volunteers and treated animals. Other preliminary studies will be conducted to determine heat and pH stability and whether or not the factor is dialysable. The purification techniques to be used on plasma from treated animals include: 1) column chromatography; 2) ethanol and $(NH_4)_2SO_4$ precipitation; 3) polyacrylamide gel electrophoresis; 4) gel filtration through Sephadex columns; 5) molecular sieving; and 6) recycling chromatography. In addition, density

gradient ultracentrifugation has been of value in achieving partial separation of other plasma compounds and will be utilized in these studies in an attempt to partially purify the plasma factor. In this work assistance has been and will continue to be given by personnel at Beckman Instrument Co. and at the Oak Ridge National Laboratory.

(3) Platelet Production Studies

(a) Peripheral Platelet Counts

For platelet counts, blood will be taken from the leg vein of rats and diluted in ammonium oxalate to lyse the red blood cells. After 20 to 30 min of mixing on an automatic rotor, a drop of diluted blood will be allowed to flow under a cover slip onto a flat, thin hemocytometer chamber and allowed to settle for 20 min in a moist evironment. Platelets will be counted under phase microscopy at 450 X by use of standard techniques.[45]

(b) Platelet Life Span

Shortening of the platelet life span has been shown to be related to smoking in a study by Mustard and Murphy.[15] Reduced life span indicates that the platelet is leaving circulation and either going into coagulation, into arterial walls or dying at a faster rate than normal platelets. If smoking shortens the life span of platelets, then one possible finding (although other hypotheses are possible) is that more young, active platelets will be in the circulation. Since younger platelets are larger and more functional than older platelets, this condition could help explain the increased rate of thrombosis among smokers. Recent unpublished results by the applicant indicate that nicotine ingestion does alter the life span of platelets.[46]

Variations in platelet life spans will be determined according to the method described previously.[47] Rats exposed to either nicotine or tobacco smoke will be given daily isotopic injections of $Na_2{}^{35}SO_4$ (1 μCi ^{35}S/gm body wt.). The animals will be killed 24 hrs after the last injection and the ^{35}S incorporation will be determined by use of a Packard Tri-Carb liquid scintillation counter. Rats will be injected with 2 ml of a 1:10 dilution of heparin (heparin sodium, 1,000 IU/ml) intraperitoneally 10 min prior to killing. A standard volume of blood will be collected into EDTA from each rat and diluted with saline to obtain a better platelet yield. The blood-saline mixture of each rat will be divided into two 15 ml conical centrifuge tubes and the platelets will be separated by differential centrifugation at 5°C. After slow centrifugation of the blood (107 x g for 30 min), the PRP-saline will be removed and the tubes spun more rapidly (760 x g for 20 min) to obtain a platelet pellet.

The platelets will be washed twice with 4 ml of saline and resuspended in 0.5 ml saline. WBC and platelet counts will be made on each platelet suspension. Two samples of platelet suspensions will be added to polyethylene counting vials each containing 10 ml of a scintillation mixture prepared in the following way: 1 liter of 1,4 dioxane; 175 gm of napthalene; 7 gm of 2,5 diphenyloxazle; and 0.375 gm of 1,4-bis-2-(4 methyl-5 phenyloxazoyle)-benezene. The radioactivity of two samples of platelet-free plasma-saline and one sample of each wash will also be determined for control purposes.

If the platelet life span is found to be altered, one must determine if the alteration is due to the platelets themselves or to plasma factors. To make this determination, animals exposed to smoke and control animals will be given ^{35}S injections as described above. Platelets from both groups will be harvested along with platelet-free plasma. Animals exposed to smoke and control animals will be injected with the labeled platelets from the opposite group and the disappearance time of the labeled platelets from the plasma of each group will be recorded. If labeled control platelets injected into smoke-treated animals disappear from the circulation faster than labeled smoke-treated platelets in control animals, then the change in life span can probably be ascribed to some plasma factor alteration rather than to alteration of the platelets. Conversely, if labeled smoke-treated platelets injected into control animals are removed from the circulation faster than labeled-control platelets in smoke-treated animals, then a platelet alteration should be considered.

(c) Platelet Size

Platelets normally decrease in size with age through a process of maturation or senescence.[48,49] Since younger platelets are larger than older platelets, the younger platelets could be more easily activated through contact with vessel walls and as a result lead to thrombus formation. Some studies have shown that the younger platelet is in fact, more functional than the older platelet.[50] The ability to determine increases in platelet size could possibly be used as a diagnostic tool in detecting potential heart attacks. If smoking causes an increase in the platelet size, then this could help explain the increase in platelet function which could help to potentiate the higher frequency of heart disorders among smokers.

Platelet size will be determined from washed platelet preparations by use of an Electrozone Celloscope attached to a 128 channel analyzer for cell sizing as in the Coulter counter method. Platelet suspensions for counting are made by diluting 5 µl of PRP into 10 ml Isoton (Coulter) to give a concentration of 1:2000. After the platelet suspensions have been counted, a value between 15,000-19,000 platelets (optimum number for sizing) will be used for sizing.

(d) Megakaryocyte Determination

Since platelets are derived from the cytoplasm of megakaryocytes,[51] one would expect relative changes in peripheral platelet numbers to be reflected by proportional changes in the megakaryocyte pool.[52] If exposure of rats to tobacco smoke causes an altered platelet life span (as measured by ^{35}S incorporation) or increases their removal from circulation by some process (as determined by size changes), then one would expect the megakaryocyte population (or a precursor cell) to be stimulated. If smoking causes a reduction in the peripheral platelet count, then one would expect megakaryocytopoiesis to be stimulated by the action of thrombopoietin.

Bone marrow samples will be obtained from the excised tibias of rats by forcing air through the bone with a needle and syringe according to the method of Jackson.[53] Megakaryocytes constitute roughly 0.1% of the total bone marrow cell population and usually are not distributed evenly in smears or squashes. Bone marrow suspensions will be collected on Millipore filters with a 0.45 μ pore size. Marrow suspensions will be prepared by adding 2-3 drops of 3.5% polyvinylpyrrolidone in saline to the marrow cells on a glass slide. After the marrow suspensions and polyvinylpyrrolidone are mixed, then 20-40 microliters will be diluted in 5 ml of saline. Two ml of this latter suspension will be forced through a Millipore filter with a syringe adaptor. The filter will be removed and allowed to dry before staining the megakaryocytes. Microscopic analysis will be used to count the megakaryocytes.

(4) Chemical Alterations of Platelets

(a) Mucopolysaccharide (MPS) Content

It has been demonstrated that increased platelet adhesiveness is correlated with an increase in the MPS of platelets.[47] Recently, nicotine injections have been shown to increase the uptake of $Na_2{}^{35}SO_4$, indicating an increase in the amount of MPS.[37] There is conflicting evidence in the literature concerning smoking and platelet adhesiveness and since MPS content is known to alter platelet adhesiveness, it is possible that smoking may alter the MPS content of platelets. Therefore, MPS determinations on platelets exposed to nicotine or smoke will allow one to correlate any MPS changes with alterations in platelet adhesiveness. Hence, increased MPS would strongly indicate potentially increased adhesiveness which could account for the increased thrombotic tendency among smokers.

MPS determination will be made following the method outlined by McDonald.[47] Rat platelets will be labeled *in vivo* by daily intraperitoneal injections (1 μCi of $Na_2{}^{35}SO_4$/gm body wt.) for 5 days before killing and determining the uptake of the isotope. At each time period the platelets of three rats will be pooled and resuspended into saline to a final volume of 2.5 ml. The radioactivity of 2 samples of the platelet suspensions, 2 samples of plasma-saline and 1 sample of each wash will be determined by use of a liquid scintillation counter as described above.

To extract MPS from platelets, duplicate 1 ml samples of ^{35}S labeled platelets will be added to 1 M NaOH and hydrolyzed for 7 hrs at 5°C with constant mixing. The samples will be neutralized with 1 ml of 4 M acetic acid. The samples will then be treated 4 times with chloroform-Amyl-alcohol reagent to remove protein. After each treatment, the samples will be centrifuged (760 x g for 10 min) and the upper layer containing the MPS will be transferred to another tube. The final supernatant fluid will be dialyzed for 40 hrs against four 1,000 ml portions of distilled water at 5°C. After lyophilization, the material will be dissolved in 500 µl of water.

For identification of specific MPS molecules, samples of extracted ^{35}S-MPS will be subjected to ascending chromatography using $MgCl_2$ (0.5 M and 1.0 M), 0.4 M $AlCl_3$ or 0.5 M $FeCl_3$ as solvents. After chromatography the samples will be washed, dried and the strips cut into three pieces which will be placed in scintillation vials containing 10 ml scintillation fluid previously described and the ^{35}S activity counted by a liquid scintillation counter. On the basis of the chromatographic method described by McDonald[47] it will be possible to identify heparin and chondroitin sulfates A, B, and C.

(b) Hexosamine Determinations

Since hexosamines (glucosamine and galactosamine) are normally present as an MPS component, one would expect to find increases and decreases in hexosamines closely correlated with changes in MPS content. If smoking causes an increase in platelet MPS, then one would expect hexosamines to be increased also. Hexosamine determinations should, therefore, give more validity to the MPS determinations.

The determination of hexosamines will be done according to the following method:[47] rat blood platelets will be collected as previously described and resuspended in 1.2 ml of physiological saline. One ml of the platelet suspension will be added to 1 ml of 2 M HCl and placed in a hot water bath (100°C) for 4 hrs to break the glycocytic and sulfate bonds of the MPS complex. After heating, the samples will be allowed to cool and 1 ml of water will be added to each tube. One ml of freshly prepared acetyl-acetone (2,4 pentanedione) mixture (1 ml of acetylacetone in 50 ml of 1 M Na_2CO_3) will be added to the samples and 0.1 to 1.0 µmole of glucosamine or galactosamine. The platelet samples will again be placed in a hot water bath (100°C) for 10 min, cooled and 5 ml of 95% ethanol will be added to precipitate protein. Next, the samples will be placed in a water bath (75°C) for 5 min after which 1 ml of p-dimethylaminobenzaldehyde (specific stain for glucosamine and galactosamine) will be added and mixed thoroughly with a Vortex mixer. The tubes will then be placed back in the 75°C water bath for 30 min with occasional shaking in order to get maximum adsorption of benzaldehyde to hexosamine resulting in a color change. After removal and cooling, the optical density (OD) of the samples will be determined at 520 mµ. The OD readings will be compared to readings of known amounts of glucosamine and galactosamine and the amount of hexosamine can then be determined from a calibration curve of OD versus hexosamine concentration.

(c) Protein Determination

Total protein determination of blood platelets will be used as a relative measure of size and therefore functional state. A younger and hence larger, more active platelet population should show more total protein per platelet.[38] If smoking or nicotine shortens the life span, then one would expect an increase in the total protein/platelet in the platelet population since more younger platelets will be in circulation.

A modified Lowry's method of protein determination will be employed.[47] One hundred microliters of platelet suspension will be added to 3.9 ml of water, mixed, and 0.5 ml of this suspension will be placed in a test tube. To each tube 5 ml of an alkaline solution (4% filtered Na_2CO_3, 2% $CuSO_4$, 4% sodium potassium tartrate 100:1:1 by volume) will be added and mixed well. The tubes will be placed in a 40°C water bath for 15 min after which 0.5 ml of Folin-Ciocalteau reagent diluted 1:2 with water will be added to each tube and mixed. After allowing 30 min for color development, the samples will be read at 660 mμ with a spectrophotometer. The OD readings for the samples will be compared to readings of known amounts of bovine serum albumin.

(5) Thrombopoietin Assays

It has been claimed that platelet life-spans are shortened due to smoking or nicotine treatment.[15] In addition a decrease in peripheral platelet count has been claimed due to smoking.[2,31] It seems possible that part of the increased function of platelets from individuals who smoke may be due to an increase in younger platelets in the peripheral blood made available by alterations in platelet production rates. Platelet production is thought to be controlled by a hormone (thrombopoietin) that is released and acts on immature megakaryocytes to increase the output of platelets in response to thrombocytopenia. Whether or not smoking causes an increase in serum thrombopoietin levels remains to be shown.

(a) Bioassay

Male C_3H mice weighing 22-25 g are used as assay recipients. The mice are injected intraperitoneally with rabbit anti-mouse platelet serum (AMPS) which is prepared and absorbed with mouse RBC as previously described.[54] Usually 0.1 ml AMPS is diluted to 0.5 ml in saline prior to injection. AMPS-injected mice with initial platelet counts (determined 4 hrs after AMPS injection by direct phase-contrast microscopy from a single drop of blood obtained by retroorbital puncture) above $50,000/mm^3$ are excluded from the assay. By use of this procedure, only 275 of 3,258 mice (8%) used in recent bioassays have been discarded. This strain of mice has a normal platelet count of about $9 \times 10^5/mm^3$. Mice are injected subcutaneously 4 times (2 times on days 5 and 6) with the first injection given 5 days after AMPS with test substance. Thirty μCi of $Na_2{}^{35}SO_4$ diluted in 0.5 ml of saline are injected intravenously on day 7 and the 24 hr radio-sulfate incorporation into platelets is determined. At the time of assay, mice are injected intraperitoneally with 0.5 ml of a heparin-Nembutal-saline solution (1.0 ml heparin, 1,000 U.S.P. units; 1.0 ml Nembutal, 50 mg; and 10 ml of saline) and platelet counts and WBC counts are made from a drop of blood obtained by retroorbital puncture. About 10 min later mice are bled from the heart into plastic syringes containing 1.0 ml of 1.0% disodium-ethylenediaminetetraacetic acid (Na_2EDTA) in 0.7% saline. The blood of each mouse is expressed into a 12 x 75 mm plastic tube and mixed with an additional 1.0 ml of Na_2EDTA. Platelets are separated from the blood by slow centrifugation (30 min at 50 x g) to obtain a platelet-rich plasma layer which is transferred into another tube and then centrifuged at a more rapid rate (15 min at 360 x g) to obtain a platelet button. Platelets from each mouse are washed with 0.5 ml of 1% ammonium oxalate, then with 1.0 ml of saline, and resuspended in 0.35 ml of Isoton (Coulter). Two 100 μl samples of the

platelet suspension are placed in plastic counting vials for the determination of radioactivity. Another sample of the suspension is used for the determination of the number of platelets in the washed platelet suspension. For platelet counting, the suspensions are further diluted (1 to 2,000) in Isoton and counted by use of an Electrozone/Celloscope (Particle Data, Inc.) with a log converter. The percent ^{35}S incorporation of the platelets is calculated:

$$\% \ ^{35}S \text{ Incorporation} = \frac{\left(\frac{\text{CPM of platelet suspension}}{\text{Platelet count of suspension}}\right)\left(\text{Body weight in grams} \times 7\%\right)\left(\text{Peripheral platelet count/ml}\right)}{\text{CPM INJECTED}} \times 100$$

(b) Immunoassay

The immunoassay utilizes a hemagglutination inhibition technique as outlined in previously published articles.[55, 56, 57] Our results indicate that TSF can be detected and quantified in sheep sera by use of this immunoassay. We also believe that this technique represents an inexpensive method for assay of TSF.

(6) Study of Platelets and Plasma Factors in Human Volunteers

Thirty ml of blood will be drawn into vacutainers containing EDTA from each volunteer and the platelets harvested as indicated above for rat platelets. Some of the platelets will be subjected to function studies. Other samples from this platelet suspension will be utilized for chemical studies as outlined above except $Na_2{}^{35}SO_4$ will not be used. Identification of MPS from unlabeled platelets will be performed as previously described.[58] The quantification is not as good as in the ^{35}S method, but is sufficiently sensitive to determine large differences. The hexosamine data, and other biochemical measurements, however, do not depend upon the use of radioactive labels. For platelet adhesiveness measurements, 2 or 3 ml of blood without an anticoagulant will be utilized. The plasma will be saved from the initial centrifugation for plasma factor studies.

The first studies will involve about 20 heavy, chronic smokers and an equal number of non-smokers to determine which, if any, of the above platelet functions or platelet chemicals are altered by smoking. The two groups will be paired for age, race, and sex. We will make every effort to screen the volunteers closely to insure that drugs known to affect some of these determinations (such as aspirin, and other agents known to inhibit ADP-induced aggregation; i.e. antihistamines, local anesthetics, antidepressants, tranquilizers, etc.) have not been used by the subjects for several days before obtaining the blood samples. In this regard, other factors known to affect platelet parameters (i.e. time after eating, diet, state of health, stress, illness, alcohol, occupation, genetic factors, and exposure to various

physical factors) will be considered as far as possible. If the scatter in physical and chemical data is widespread, then it will be necessary to investigate the health and habits of each volunteer more closely. The clinical hematology group headed by Dr. S. Krauss and located in this research center will collaborate in such studies if the situation demands it. If the data are not widespread, many of the chemical or physical factors mentioned may not need to be considered in greater detail.

Based on results noted in the study of heavy smokers, acute experiments will be carried out using groups of volunteers in a fasting state to consider the effects of smoking on platelets following smoking of 2-4 cigarettes in about 2 hrs. Some of these volunteers will be non-smokers, others will be subjects who have smoked in the past while still others will be current smokers with different smoking habits. Control subjects (roughly matching the categories just mentioned) will also be utilized in this study. These control subjects will provide data on which to determine the effects of obtaining several aliquots of 30 ml of blood in the short time period contemplated. Blood samples will be taken before smoking, following smoking, and at intervals thereafter to establish the rates at which platelet function or chemical makeup are reversible when smoking is curtailed. We plan to analyze the platelet suspensions and plasma collected in these acute studies in the same manner as outlined above for the studies in chronic smokers. All studies using human subjects will be carried out in conformity with directives issued by our local committee on human welfare and experimentation to whom the protocol is being considered for approval. Appropriate forms for obtaining informed consent [i.e. (1) summary of explanation to patient for obtaining informed consent for experimental procedure; (2) consent for experimental procedure; and (3) forms required by The Council for Tobacco Research] of volunteers will be used. Copies of these forms are attached to this proposal.

(7) Phasing of Study

We plan to organize our research activities as follows: the first year will be devoted to animal studies designed to determine the platelet and/or plasma alterations, which are responsible for the hypercoagulability state found among smokers. Animal studies are specifically needed in order to maintain the highest level of control possible. In addition, experiments involving the use of isotopes are not possible in human subjects; therefore, certain platelet alterations such as production or exact MPS alterations necessitate animal models. If a plasma factor alteration is indicated, as our preliminary studies have shown, then quantification of several plasma proteins will be determined.

The second year will involve continued animal experimentation combined with human volunteers. These investigations will allow one to determine if certain changes in platelet activity of animals are analogous to those found in humans.

The third year will be devoted to determining if the alterations in the platelets and/or plasma from animals exposed to smoke or nicotine are similar. In this terminal year we plan to screen human volunteers with the goal of demonstrating similar platelet and/or plasma alterations in humans who smoke as those found in animals where applicable. Some smokers have a significantly higher incidence of cardiac dysfunction as compared to the non-smoking population. It is hoped that the alterations found in these smoking volunteers will enable our group to diagnose a potential cardiac patient prior to the onset of the malfunction. Therefore, in order to determine a broad spectrum of platelet and/or plasma alterations found among smokers a combined study involving animal models and human volunteers needs to be undertaken.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Ample physical facilities are available for the principal investigator and co-investigator to conduct the proposed research project at the University of Tennessee Memorial Research Center and Hospital. These facilities include: adequate laboratory work space, a cold room, and a fully accredited animal facility. Although some of the equipment lised below is used by several programs, it is available on a part-time basis. These items include: a refrigerated centrifuge, a freezer, phase microscope, Model A Coulter counter, Model 3375 Tri-Carb Liquid Scintillation Counter, photographic equipment, analytical balance, and lyophilizer. In addition, a celloscope with a 128 channel analyzer, a Chrono-log aggregometer, and a Fisher Autocytometer are available. A new well-equipped medical library has been established in the Memorial Research Center. Excellent library facilities at the Oak Ridge Associated Universities and at the Oak Ridge National Laboratory are readily available.

Senior members of the staff of the Memorial Research Center are available for consultation and are currently utilizing several research methods which would be applicable to the studies proposed in this application. The members of the staff and their fields of interests are: Dr. R.D. Lange, erythropoietin and use of the HAI technique for detection of ESF; Drs. A.I. Chernoff, J.F. Fuhr, and D. Dupourque in amino acid analysis, identification of abnormal hemoglobins, and investigation on the effect of smoking on the stability, oxygen affinity, and binding of carcinogenic ligands to hemoglobin; Dr. W. Porter in glycoprotein structure; Drs. P.W. Wigler and W.R. Farkas in RNA enzyme chemistry; Dr. A.L. Kretchmar in stem cell repopulation and mathematical models of hematopoiesis; Dr. T.J. Yan in immunology; Dr. A. Solomon in immunoglobulin structure; Dr. B.B. Lozzio in RES function and lymphocyte stimulation by phytohemagglutination; Dr. C.B. Lozzio in cytogenetics; and Dr. S. Krauss in white blood cell function in disease. In addition, Dr. T.T. Odell, Jr. of the Biology Division of Oak Ridge National Laboratory is available for discussion of work on platelets and their functions.

11. Additional facilities required:

NONE

12. Biographical sketches of investigator(s) and other professional personnel (append):

SEE ATTACHED

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

SEE ATTACHED

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(25-35 deleted)

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
P. McDonald, Ph.D., Research Associate Prof.	25	5,000
Marshall E. Reese, Jr., Ph.D. Research Associate	100	10,000
Technical		
Rose Clift, Technician	100	7,000
Ann Beardsley, Secretary	10	595
Bill Wolfenbarger, Lab Aide	50	2,315
		24,910
Fringe Benefits (12% of S&W)		2,989
	Sub-Total for A	27,899

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B. Consumeble supplies (by major categories)	
Animal Charges	3,450
Glassware, hardware, plasticware	500
Chemicals, syringes, disposable supplies and cigarettes	900
Isotopes	500
Sub-Total for B	5,350

C. Other expenses (itemize)	
Service contracts, maintainence	800
Computer time	500
Office supplies, xerox, photography	400
Publications and printing	200
Travel	600
Sub-Total for C	2,500
Running Total of A + B + C	35,749

D. Permanent equipment (itemize)	
Smoking machine	2,000
Updating existing instrument to dual channel aggregometer	500
Calculator (Marchant Model 730)	300
Sub-Total for D	2,800
E	5,362
Total request	43,911

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	29,573	5,850	3,000	300	5,763	44,486
Year 3	31,347	6.350	3,500	-0-	6,180	47,377

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14. Explanation of Budget:

Consumable supplies:

Animal charges: Current animal charges for rats are: $2.50 each purchase price and $2.50/rat/month care. 600 rats will be needed the first year - $3,000; 300 mice for TSF bioassays will be needed; each mouse costs $1.00 and care is $.50/2 months/mouse, $450; $Na_2{}^{35}SO_4$ is used as a platelet label. This isotope costs $50/5 mCi.

Computer time is calculated at rates currently available to this institution. Time includes program, consultation and running time.

Travel:

To send the principal investigator to one meeting and the co-investigator to two meetings dealing with platelet function and smoking.

Equipment:

A new smoking machine costing about $2,000 is needed in this work. The machine that we are currently using is adequate for our preliminary studies, but several problems inherent to its design exist. First, smoke is forced through restricted openings which allow the build-up of coacervate smoke particles at an extremely rapid rate, thus decreasing the amount of smoke available for inhalation. Another portion of the standard puff (15-20%) is trapped in the cigarette holder before the smoke reaches the exposure chamber, again reducing the actual exposure. The most serious design problem with our machine is the need for immediate mixing of the fresh smoke with air which would greatly reduce coacervate formation as well as providing a consistent smoke-air mixture for inhalation. At present the animals are rotated systematically in order to insure a more even dose of smoke. Only 5 rats can be exposed at one time in our machine, the modified Walton will allow us to increase this number thereby increasing our sample size.

Updating the existing aggregometer to a dual channel instrument is needed in order to measure the large number of platelet samples planned. The dual channel updating on the aggregometer will allow us to aggregate the samples more rapidly as well as allowing for larger sample sizes. As Levine[23] recently indicated, aggregations need to be performed within 30 min after bleeding. The dual channel attachment will greatly reduce the lag time between aggregations.

Salaries:

For years 2 and 3 the salaries are computed at 6% increase per year.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Platelet Function and Smoking	American Medical Association Education Research Foundation	87,097	5/1/71 to 4/30/74
Characterization and Immunoassay of Thrombopoietin	American Heart Association	53,680	7/1/72 to 6/30/75
Production, Purification and Assay of Thrombopoietin	U.S. Atomic Energy Commission	33,000	6/1/73 to 5/30/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Thrombopoietin: Immunoassay and Characterization	National Institute of Health Approved but not funded #HL-14637-01A2	121,993	

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Ted P. McDonald, Ph.D.

Signature Ted P. McDonald Date 12-14-73

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Responsible officer of institution

Typed Name Hilton A. Smith

Title Vice Chancellor for Grad. Studies & Res.

Signature Hilton A Smith Date 12/17/74

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